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The Development of a Radioimmuno-Assay for Carcino-Embryonic Antigen with some Applications

Clinical Evaluation of Cercino-Embryonic Antigen, I

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Summary: This paper, the first in a series devoted to the study of the clinical usefulness of estimations of carcino-embryonic antigen (CEA) in serum and urine, describes in detail a direct radioimmunoassay for CEA in serum and urine (a modified *Egan* technique, *Egan, M. L.* et al. (1972), Immunochemistry, *9*, 289–299). A comparative study of the behaviour of CEA batches and anti-CEA antisera from different laboratories in the radioimmunoassay is presented.

The incidence of increased serum CEA levels in healthy smokers was found to be related to smoking habits.

Assays of CEA in serum obtained by the direct technique and the *Hansen* perchloric acid – zirconyl gel technique (*Hansen, H. J.* et al. (1971), Clin. Res. *19*, 143–147) showed comparable results.

Various problems affecting the assay of CEA in serum and urine are critically discussed, e.g. criteria for absorbing-out of anti-CEA antisera, identification of CEA, factors causing false CEA values, use of reference materials, acceptability of a strict cut-off level to indicate increased CEA levels, and factors governing the choice of antisera. In this connection we present a new approach which maintains a reliable and consistent cut-off level in follow-up studies.

Evidence is presented that urinary CEA is heterogeneous and perchloric acid-unstable.

Entwicklung eines Radioimmunassay für carcino-embryonales Antigen und seine Anwendung
Klinische Bedeutung des carcino-embryonalen Antigens, 1. Mitteilung

Zusammenfassung: Diese erste Arbeit einer Serie, die sich mit der klinischen Bedeutung der Bestimmung von „Carcino-embryonic antigen" (CEA) im Serum und im Urin befaßt, beschreibt eine direkte radioimmunologische Bestimmung für CEA im Serum und im Urin (modifiziert nach *Egan, M. L.* et al. (1972), Immunochemistry, *9*, 289–299). Eine Vergleichsstudie bezüglich der CEA-Präparationen und Antisera gegen CEA aus verschiedenen Laboratorien wird beschrieben.

Es wird gezeigt, daß erhöhte CEA-Werte bei gesunden Rauchern mit den Rauchgewohnheiten zusammenhängen.

Bestimmungen von CEA im Serum mit der direkten radioimmunologischen Methode und der Perchlorsäure-Zirconyl-gel-Technik (*Hansen, H. J.* et al. (1971), Clin. Res. *19*, 143–147) weisen vergleichbare Ergebnisse auf.

Kritisch besprochen werden mehrere Probleme, die mit der CEA-Bestimmung zusammenhängen, wie z. B. das Problem der Isolierung von CEA-Antiseren durch Absorption, Charakterisierung von CEA, Faktoren, welche falsche CEA-Werte verursachen, Gebrauch von Referenzsubstanzen, Zuverlässigkeitskriterien für einen Grenzwert zwischen dem normalen und erhöhten CEA-Bereich, und Faktoren, die die Wahl von Antisera bestimmen. In diesem Zusammenhang wird ein neues Verfahren vorgeschlagen, um mit zuverlässigen Grenzwerten während der Verlaufsstudie arbeiten zu können. Es wird nachgewiesen, daß CEA im Urin heterogen ist und Extraktion mit Perchlorsäure zum Verlust von nachweisbarem CEA führt.

Introduction

Studies by *Gold & Freedman* (1, 2) in 1965 revealed the presence of a tumour-associated antigen in adenocarcinomas of the colon as well as in foetal colonic mucosa. This antigen was therefore named carcinoembryonic antigen (CEA)[1]) of the human digestive system.

In 1969 *Thomson* et al. (3) introduced a radioimmunoassay for the measurement of a colonic tumour-associated antigen in human sera.

The usefulness of serial measurements of CEA in monitoring the effects of therapy and in the long term follow-up has attracted more attention very recently. *Mach* et al. pointed out that, despite the large number of CEA tests already performed in many institutes (4–11), very few follow-up studies have been reported (12). A more extensive study of the value of CEA in different body fluids for diagnosis, follow-up and prognosis in cancer patients was started in 1973. The generally accepted assay for CEA is the radioimmunoassay (13, 14, 15). CEA is a glycoprotein soluble in 1 mol/l perchloric acid (16).

Other perchloric acid soluble glycoproteins, like non-specific cross reacting antigen, which have antigenic determinants in common with CEA, have been reported (17–22). Blood group antigens, such as A, etc., can be associated with certain CEA preparations (23, 24) and in consequence the antiserum may contain a high titer of anti-A antibodies. Anti-A antibodies, either from the antiserum or from the test sample, may become attached to the labelled CEA in the assay and thus diminish the accessibility of the anti-CEA antibodies for the labelled CEA. In such cases the test results are unreliable. If a sample contains blood group antigen A, an interaction can take place between A, the A-like site on labelled CEA and anti-A antibodies present in the anti-CEA antiserum. A radioimmunoassay for CEA will also measure antigen A activity and the test result is not representative of the true CEA content of the sample. This is accounted for in the technology of the radioimmuno-assay used in the clinical study in the present paper.

Materials and Methods

Reagents

Phosphate buffers

Sodium phosphate-buffered saline contained NaCl (9 g/l) and dipotassium ethylene dinitrilotetra-acetate (0.7 mmol/l) (K_2EDTA) in phosphate buffer (0.05 mol/l). The resulting solution had a pH of 7.4. In some cases 0.5 ml rabbit serum was added to 100 ml of this solution.

Borate buffer

Sodium borate (0.125 mol/l), pH 8.4.

Radioactive iodine (^{125}I) was obtained from the Radiochemical Centre (code IMS-30).

Chloramine T solution

Chloramine T (Merck) was dissolved in borate buffer (0.8 g/l).

Sodium metabisulfite solution

Sodium metabisulfite (Merck) was dissolved in borate buffer (2.4 g/l).

Isolation of CEA

Isolation of CEA was performed, using essentially the procedure described by *Krupey* et al. (25), with several modifications. The perchloric acid extract of liver metastases from a colonic tumour was passed through a Sepharose 4B column. Phosphate buffer (pH 5.5) containing 1 g/l NaN_3 was used for preparation of the column and for elution. The fractions were monitored by absorbance measurements at 280 nm and tested for the presence of CEA by the *Ouchterlony* technique, using anti CEA antiserum kindly provided by Dr. *S. von Kleist* (Ville-juif, France). The CEA-containing fractions were pooled, lyophilized and re-run on Sepharose 4B. The CEA-containing fractions were dialysed carefully against twice distilled water until the Na and K contents were zero (as judged by flame-photometric determination), then lyophilized. The CEA thus prepared, and used in this study, is designated 2SDI.

Preparation of standards

A stock solution of about 20 mg/l was prepared in phosphate buffered saline, containing rabbit serum and stored at – 20°C. Weighing was done with a *Cahn* microbalance. Dilutions were made with phosphate buffered saline, containing rabbit serum, to 1000 and 750 µg/l for the assay in serum and to 500 and 200 µg/l for the assay in urine.

Labelling of CEA

CEA (about 300 µg) was dissolved in twice distilled water (first brought to neutrality with ammonia) to a concentration of 1 g/l. Quantities of 10 µl were dispensed in a number of tubes and lyophilized. The tubes were kept at – 20°C until iodination.

The first step in the iodination procedure was the addition of 20 µl phosphate buffered saline to a tube brought to room temperature.

Radioiodination of CEA, using 1 mCi ^{125}I, was accomplished by the *Brown & Reith* modification of the *Hunter & Greenwood* procedure (26) within 2 days after receipt of ^{125}I. The iodination time was usually 50 seconds. The iodination reaction was stopped by addition of 25 µl bisulfite solution and the mixture was transferred to a Sephadex G 25 column previously equilibrated with phosphate buffered saline containing rabbit serum. The transfer was facilitated by addition of 0.2 ml KI solution (carrier iodide).

The column was slowly eluted with phosphate buffered saline and the fractions containing the highest activity were used for the radioimmuno-assay. As the labelled CEA decayed, the percentage of labelled CEA bound by the diluted anti-CEA antisera gradually decreased (about 10% in 3 weeks). As a rule, freshly labelled CEA was applied every 2–3 weeks.

Anti-CEA antisera

Various goat antisera were used: NKI-3 was prepared in goats by intramuscular injections of 100 µg 2 SDI in physiological saline, emulsified with complete *Freund*'s adjuvant. Injections were repeated 5 times at intervals of about 1 month. The antiserum with the highest titre of anti-CEA antibodies was chosen for use in the clinical studies and was named NKI-3[2]).

[1]) *Abbreviations*

Ace = anti-CEA antiserum prepared by Dr. *Todd*'s group (Duarte, USA); CEA = carcino-embryonic antigen; CEA_{DU} = CEA prepared by Dr. *Todd*'s group; CEA_L = CEA prepared by Dr. *Mach*'s group (Lausanna); G-10-2 = anti-CEA antiserum prepared by Dr. *Mach*'s group; 6G3 = anti-CEA antiserum prepared by Dr. *Neville*'s group (London); NKI-3 = anti-CEA antiserum prepared by us; 2SDI = CEA prepared by us.

[2]) Small amounts of NKI-3, sufficient for 1000 assays, are available on request.

G-10-2 was a gift from Dr. *Mach* (Lausanne). It had been absorbed out with polymerized human normal serum and normal perchloric acid extractable glycoproteins.

Antisera to goat serum

These were obtained from the Central Laboratory of the Netherlands Blood Transfusion Service (Amsterdam).

Procedure for the radioimmuno-assay

Principles of the assay

CEA levels were estimated by the double antibody technique of *Egan* et al. (15) with several modifications.

Two assay systems have been developed: system 1 for assay of CEA in serum, system 2 for CEA measurements in urine.

Radioimmuno-assay in serum (system 1)

Plastic tubes (size 4 cm, i.d. 8 mm with round bottom) were used for the assay. An amount of 200 µl of the patient's serum was mixed with 50 µl goat antiserum to CEA diluted with phosphate buffered saline containing rabbit serum, and 20 µl phosphate buffered saline containing rabbit serum. For NKI-3 a dilution 1:12.000 was generally used. Incubation was performed overnight in a water bath at 37°C; 50 µl [^{125}I]CEA (3 µg/l) were added to each tube and incubation was then continued for two hours. Separation of antibody-bound CEA was performed as described under "Separation of antibody-bound CEA from unbound CEA". For the construction of the standard inhibition curve serial two-fold dilutions (with phosphate buffered saline containing rabbit serum) were prepared from two different CEA stock solutions containing 1000 and 750 µg/l CEA in phosphate buffered saline containing rabbit serum, respectively, to obtain more points for the construction of the standard inhibition curve. The procedure as described above was followed with two modifications: 200 µl of the patient's serum was replaced by 200 µl normal human serum and 20 µl phosphate buffered saline was replaced by 20 µl of a diluted standard solution. The normal serum used in the preparation of the standard curve was checked for CEA content by reference to a "standard pool". For routine control, pooled sera were prepared and aliquots were stored at – 20°C.

In every assay run, four different pools covering the whole range of measurable CEA values were incorporated. Statistical evaluation was accomplished by calculating the mean value for a period of several weeks and the 95% confidence limits. The results of assay routinely performed in one run were accepted only if at least 3 out of 4 pools showed values within these limits.

Radioimmuno-assay in urine (system 2)

Urine samples were dialysed against phosphate buffered saline for 3 hours at 4°C while stirring, and stored frozen until assay. The incubation tubes (plastic tubes; size 4 cm, i.d. 8 mm with round bottom) contained 100 µl dialysed urine and 100 µl antiserum diluted with phosphate buffered saline containing rabbit serum. After overnight incubation at 37°C, 50 µl labelled CEA solution (3 µg/l) were added. Incubation was continued for 2 hours. The contents of the tubes were then subjected to the procedure as described for the assay in serum. For the construction of standard inhibition curves, aliquots (100 µl) of diluted CEA solutions were used. Again, two different stock solutions were used for the preparation of serial dilutions in phosphate buffered saline containing rabbit serum. For control a CEA solution in phosphate buffered saline containing rabbit serum was prepared and aliquoted. Aliquots were thawed and run with every series of routine samples. The inter-assay variability of one control over a period of 6 months was 8.8% (mean 54 µg/l, n = 26).

Titration of antibody

As a control after each radio-iodination, antiserum was titrated and the shape of the titration curve was estimated. The concentration giving about 75% of the maximal precipitation of radioactivity was chosen.

For system 1, 50 µl portions of six dilutions (usually from 10^{-2} down to 3×10^{-5} in steps of 1:3) of antiserum were added to 0.2 ml human normal serum. Then, 50 µl of [^{125}I]CEA was added. The reaction mixture was incubated for 3 hours at 37°C.

For system 2, tubes were used containing 100 µl diluted antiserum (in phosphate buffered saline containing rabbit serum), 100 µl phosphate buffered saline containing rabbit serum and 50 µl iodinated CEA (3 µg/l). The tubes were incubated for 2 hours at 37°C.

Separation of antibody-bound CEA from unbound CEA

This procedure was followed in system 1, system 2 and in the titration of antibody; 50 µl normal goat serum at appropriate dilution (with phosphate buffered saline containing rabbit serum) followed by 50 µl rabbit anti-goat serum (usually diluted with phosphate buffered saline containing rabbit serum) were added. The tubes were stored overnight at 4°C.

After addition of 0.3 ml phosphate buffered saline centrifugation was carried out. The supernatant was carefully removed by aspiration. The residue and supernatant were then counted separately. Quantitation of the appropriate dilution of the precipitating antiserum and the normal goat serum with respect to the applied dilution of the anti-CEA antiserum was achieved by a checkerboard titration procedure.

Comments

Chemical characterization of 2SDI

The amino acid and monosaccharide composition of the CEA preparation is given in table 1.

Immunological characterization of 2SDI by immuno-diffusion

Figure 1 shows complete identity between 2SDI and a reference CEA prepared by Dr. *Searle* (Charing Cross Hospital, London). The anti-CEA antiserum applied in

Tab. 1. Amino-acid and carbohydrate composition of 2SDI. For comparison, data on CEA prepared by Dr. *Egan* (Duarte, California) are given.

Amino acid[1])	2SDI	CEA_{Du}
lysine	2.1	2.8
histidine	1.6	1.7
arginine	3.4	3.1
aspartic acid	14.8	15.7
threonine	8.7	8.7
serine	10.3	11.4
glutamic acid	10.8	9;6
proline	7.8	8.0
glycine	5.7	6.3
alanine	5.7	6.2
cysteine	2.1	1.6
valine	6.9	6.6
methionine	0.1	0.4
isoleucine	3.9	3.7
leucine	8.7	7.6
tyrosine	4.8	4.2
phenylalanine	2.5	2.2
monosaccharide[2])		
fucose	7.2	9.2
mannose	7.2	9.3
galactose	10.0	8.9
N-acetyl-galactosamine	0.6	1.7
N-acetyl-glucosamine	16.7	15.7
sialic acid	2.6	variable

[1]) mole/100 mole
[2]) % by weight

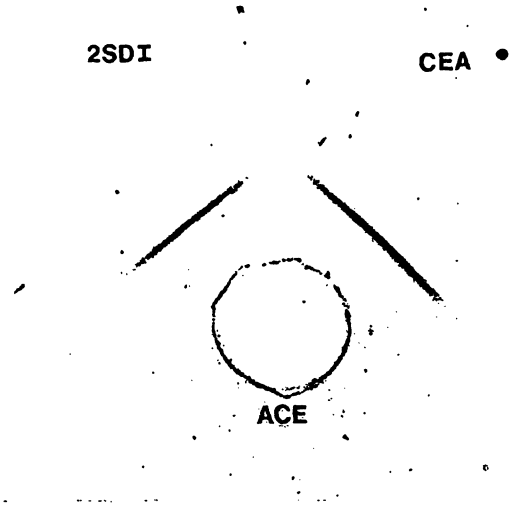

Fig. 1. Comparative radial immunodiffusion. For explanation see text (Magnification 4.4x).

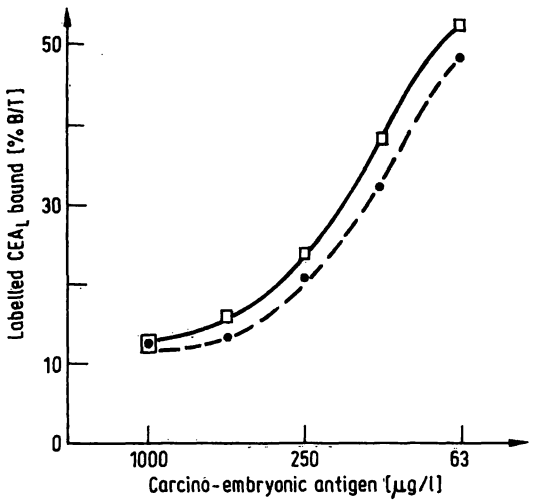

Fig. 2. Comparison of standard inhibition curves (system 2) obtained with doubling concentrations of CEA_L (□—□) and 2SDI (●– –●). Antiserum: anti-CEA G-10-2, diluted 1/10 000.

the *Ouchterlony* plates was prepared by the *Todd* group (Duarte, California) and named Ace 17 (27). A completely identical result was obtained with an unabsorbed rabbit anti-CEA antiserum. No spur was observed in both cases.

Immunological characterization of 2SDI and NKI-3 under radioimmunoassay conditions

In order to check the antigenic identity of 2SDI, other preparations of CEA and anti-CEA antisera were compared with the preparations used in assay system 1 or assay system 2. For this purpose different standard inhibition curves were prepared, in which unlabelled or labelled CEA or anti-CEA antiserum was replaced by a similar preparation kindly provided by other groups.

Standard inhibition curves obtained in system 2 by serial dilutions of CEA_L and dilutions of 2SDI were parallel (fig. 2). Both experiments were performed using labelled CEA from the Lausanne group.

Figure 3 compares a standard inhibition curve (system 1) with a standard curve obtained by serial dilution of CEA prepared by Dr. *Gold*'s group (Montreal). The other reagents, such as labelled 2SDI, antiserum NKI-3 and normal serum, were identical for both curves. A nearly identical behaviour of 2SDI and the CEA from the Montreal group is evident from this figure.

Figure 4a shows the inhibition lines for 2SDI and CEA supplied by *Todd*'s group (Duarte, California) in an assay (system 2) based on the anti-CEA antiserum Ace. In two out of three experiments labelled CEA was prepared from 2SDI, in one experiment from CEA supplied by *Todd*'s group. It will be noted that the different curves are superimposable except for the range above

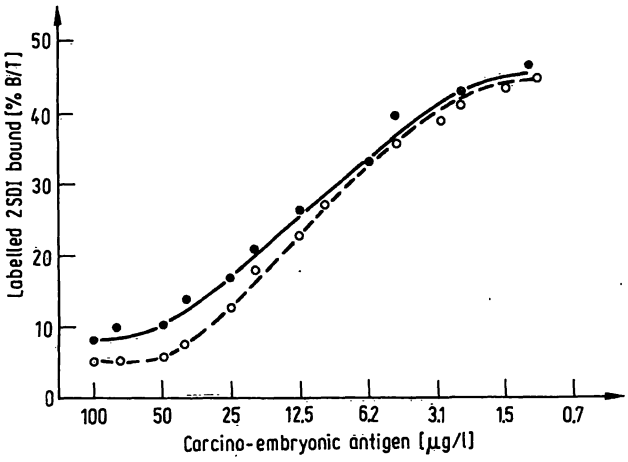

Fig. 3. Comparison of standard inhibition curves (system 1) obtained with CEA prepared by Dr. *Gold*'s group (●—●) and 2SDI (○—○). Antiserum: anti-CEA NKI-3, diluted 1/16 000.

32 µg/l. A similar experiment, but based on the anti-CEA antiserum NKI-3, is described in figure 4b. Replacement of unlabelled and labelled 2SDI by the corresponding reagents of *Todd*'s group yields a practically identical curve.

A study of the binding-inhibitory activity of non-specific cross-reacting antigen in the radioimmunoassay of CEA was made as follows. A standard inhibition curve was obtained as described under "Materials and Methods" for the assay in serum. In an otherwise identical experiment, serial dilutions of 2SDI were replaced by solutions of non-specific cross reacting antigen kindly provided by Dr. *Neville* (London). The results

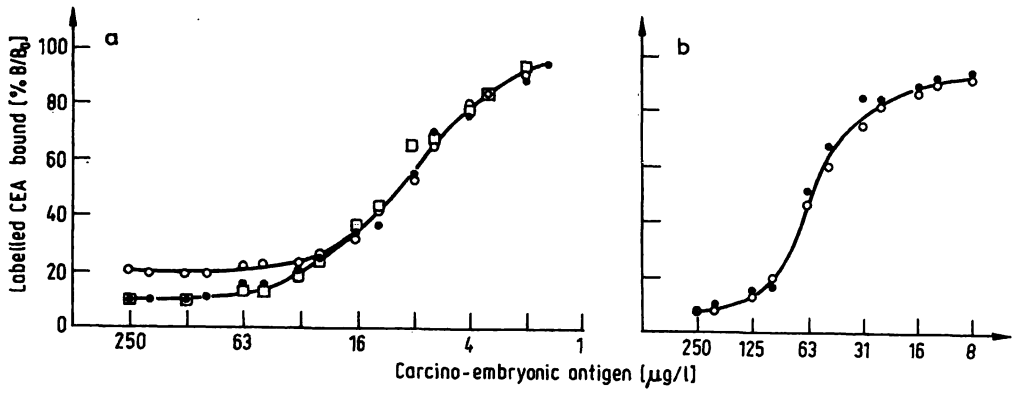

Fig. 4. Comparison of standard inhibition curves (system 2). Antisera: anti-CEA Ace, diluted 1/3200 (a), and anti-CEA NKI-3, diluted 1/21 000 (b).
(a) binding of CEA_{DU} and [^{125}I] 2SDI (o—o); CEA_{DU} and [^{125}I] CEA_{DU} (□—□); 2SDI and [^{125}I] 2SDI (•—•); (b) binding of CEA_{DU} and [^{125}I] CEA_{DU} (•—•); 2SDI and [^{125}I] 2SDI (o—o).

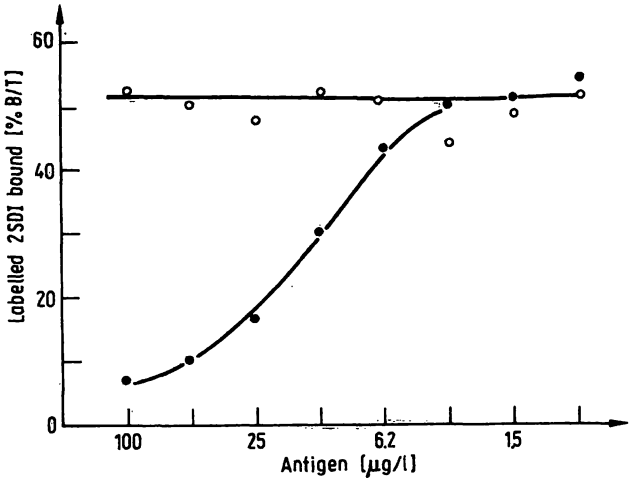

Fig. 5. Inhibition of binding of labelled CEA ([^{125}I] 2SDI) by CEA (2SDI, •—•) or by non-specific cross reacting antigen (o—o) in system 1. Antiserum: anti-CEA NKI-3, diluted 1/12 000.

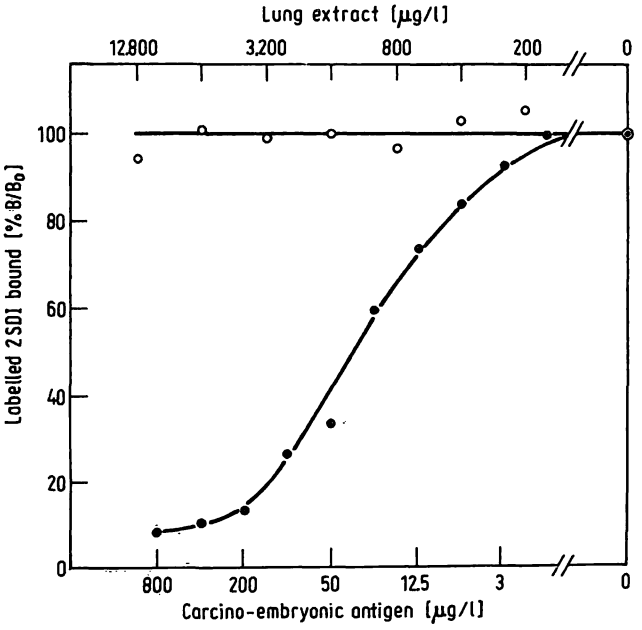

Fig. 6. Inhibition of binding of labelled CEA ([^{125}I] 2SDI) by CEA (2SDI, •—•) or by perchloric acid extract of normal lung (o—o) in system 2. Antiserum: anti-CEA NKI-3, diluted 1/30 000.

(fig. 5) show that non-specific cross reacting antigen does not interfere in the assay. Identical results were obtained in the absence of normal serum (non-specific cross reacting antigen tested to 500 µg/l). In the same way, non-specific cross reacting antigen did not replace labelled 2SDI bound to G-10-2. The specificity of NKI-3 was further tested by adding up to 12,800 ng perchloric acid extract of normal lung in the assay. No significant inhibition by perchloric acid-soluble glycoproteins was found (fig. 6).

On the other hand, normal serum causes decreased binding of labelled CEA, as demonstrated in figure 7. Since the binding curves show non-parallelism, non-specific proteins may be largely responsible for the decrease observed.

In another experiment, iodinated 2SDI was incubated with several blood group antisera as described by *Holburn* et al. (24). Saturated ammonium sulphate was then added and the precipitate and supernatant were counted to determine the percentage of bound labelled 2SDI.

Precipitated non-specifically bound [^{125}I]CEA was determined by adding buffer instead of antibody solutions.

Whereas anti-CEA antiserum did bind 92.0% of the labelled 2SDI and non-specifically bound 2SDI was

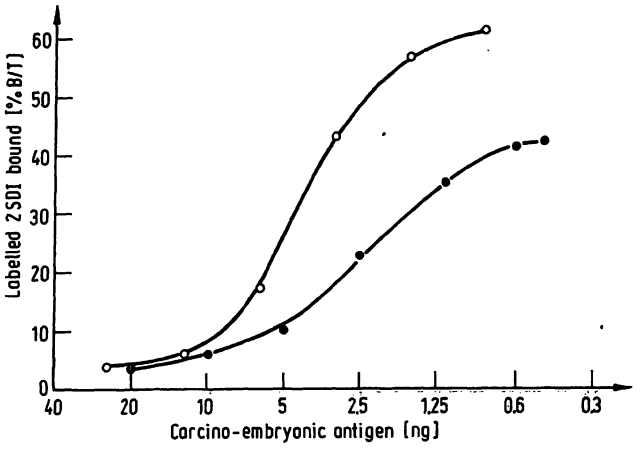

Fig. 7. Comparison of standard inhibition curves obtained with [^{125}I] 2SDI in the presence of normal serum (•—•, system 1) and in absence of normal serum (o—o, system 2). Antiserum: anti-CEA NKI-3.

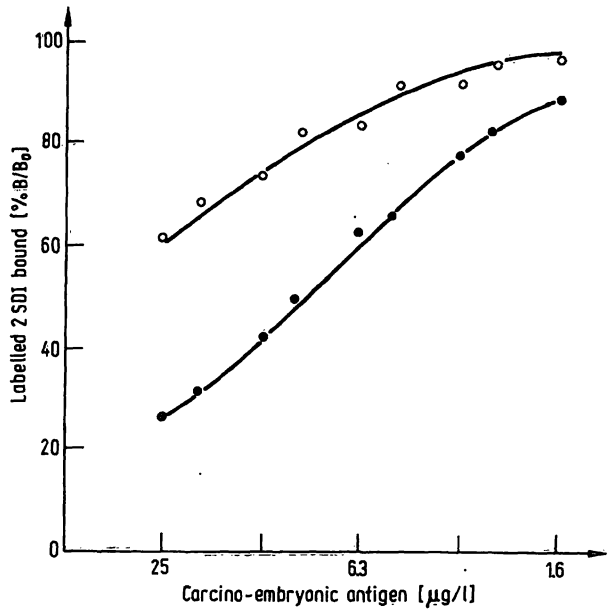

Fig. 8. Comparison of standard inhibition curves obtained by addition of labelled CEA ([^{125}I] 2SDI) to the assay mixture (system 1) simultaneously with the other reagents and incubation overnight (o—o) or according to the procedure described in "Materials and Methods". Antiserum: anti-CEA Ace, diluted 1/2700.

8.8%, binding by anti-A or other anti-blood group antibodies was in the range of 9.1–14.0%. These results indicate no significant association with blood group substances.

Storage of reagents

It was observed that antisera, if stored frozen (undiluted or diluted), retain their original binding activity even after two years of storage. The binding capacities of frozen dried NKI-3 made up with twice distilled water after storage for 2 years, were unchanged.

It has been reported (28) that CEA loses its potency to compete with labelled CEA for the binding sites if stored in the frozen dried state for some months. However, a solution of 2SDI kept frozen for more than one year and a solution freshly prepared from 2SDI, stored frozen dried for the same period showed identical standard inhibition curves.

Methodological validation

Delaying addition of the labelled CEA as one of the modifications of the *Egan* procedure considerably improved the sensitivity of the assay. An illustration is given in figure 8. Overnight incubation for the formation of the antibody complex was performed for practical reasons. Similar results were obtained with NKI-3 and 2SDI.

The standard pool

The percentage of bound to total [^{125}I]CEA (%B/T) was determined for sera from several healthy controls (non-smokers). At the same time a standard inhibition curve with NKI-3 was prepared, but according to system 2, thus omitting normal serum. The results were quantitated using this standard curve. It is realized that the CEA contents thus measured are over-estimated but in a reproducible way (see fig. 7). The normal serum showing the lowest CEA level was regarded as "zero-serum" by definition. Next, a pool serum (about 10 µg/l) was prepared. After careful mixing the standard pool was aliquoted and stored at – 20°C. Aliquots of the standard pool were analysed (system 1) for CEA under various conditions, i.e. using different batches of [^{125}I]CEA of varying age (up to 3 weeks). The mean value was 8.9 µg/l ± 0.6 (n = 24). For the standard pool a value of 9.0 µg/l was assessed. A fresh batch of normal serum was accepted *only* if, with the corresponding standard inhibition curve, a value for the standard pool was obtained which lay between 8.0 and 10.0 µg/l. If a value below 8 µg/l was found, the normal serum contained an unacceptable amount of CEA.

Results

Assay in serum

With the value of 3 µg/l a fall of approximately 10% in the percentage binding was achieved.

Therefore 3.0 µg/l value was used as the cut-off point in most assays. For the ranges of 30–40, 16–24, 10–14 and 4–6 µg/l the coefficients of variation were 7.2, 6.2, 8.2 and 12.1% respectively.

Sera from 115 apparently healthy men and women were found to contain less than 3.0 µg/l. The youngest individual was 18 years old and the oldest was 65.

Of the 115 controls, 35 admitted that they smoked regularly (at least 10 cigarettes or 4 cigars per day). Out

of these 115 controls, 36 had blood group 0RhD+, 12 had blood group 0RhD-, 25 had blood group ARhD+, 6 had blood group ARhD-, 8 had blood group BRhD+ and one had blood group ABRhD+. The blood group of the remaining 27 controls was unknown. Serum from six other healthy persons contained 4.0 to 6.0 µg/l. All but one smoked regularly. This result prompted us to establish the normal range in 49 healthy persons, qualified as blood donors and regularly smoking 15 or more cigarettes per day. The age of the smokers varied from 19 to 65 years with a mean value of 35. Of these 49 persons, 14 were taking oral contraceptives and 4 received medication (tranquillizers, etc.). The results are given in table 2.

Two sera, obtained from healthy persons with a high titre of blood group A, were also tested for CEA content. The result was in both cases < 3 µg/l. A group of random sera of eighty-five patients suffering from malignant diseases were analysed according to the *Hansen* technique (29, 30).

Figure 9 compares the results obtained by the two methods. The values between the limits of detection gave the regression: y = 1.30 x + 0.68, r = 0.8908. A standard inhibition curve obtained with Hoffmann LaRoche reagents and one obtained with the same reagents but replacing CEA by 2SDI were completely superimposable.

Assay in urine

Clinical studies first concerned patients with bladder carcinoma. At that time the only anti-CEA antiserum available to us was G-10-2, and this antiserum was adopted as a routine.

A representative curve is shown in figure 10. Results lower than 30 µg/l were not quantitated but expressed at ≤ 30 µg/l. In a series of 20 healthy persons (predominantly males) values not exceeding 30 µg/l were found. Since problems arose when determining urinary CEA levels in normal females, the normal range will be dealt with in another communication (part 2 of this series).

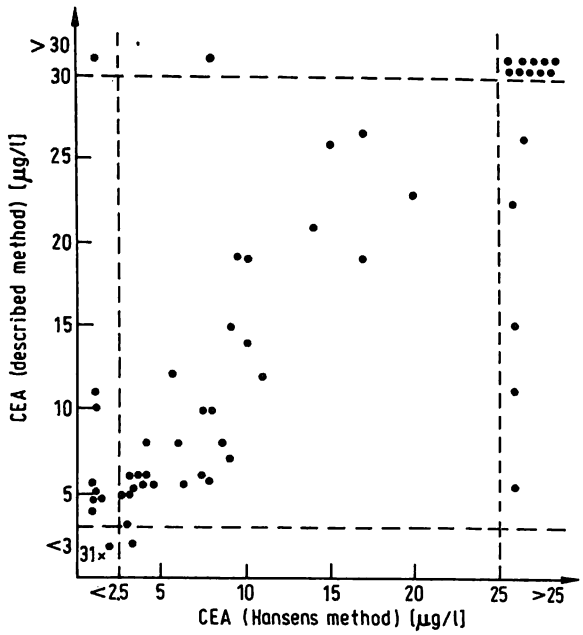

Fig. 9. Comparison of serum CEA levels measured by our and by the *Hansen* method.

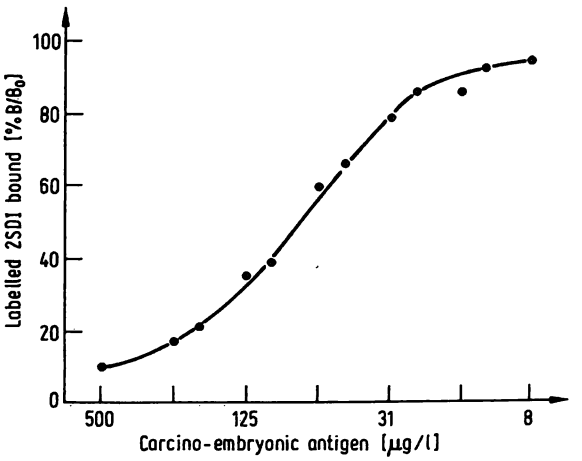

Fig. 10. Standard inhibition curve according to system 2, representative of those obtained in our laboratory. Antiserum: anti-CEA G-10-2, diluted 1/10 000.

Tab. 2. CEA levels in smokers aged 19–65 years. The figures denote number of smokers.

Group		CEA titre (µg/l)		
smoking habit since	Total no.	< 3	3–6	6–9
0.2– 2 years	4	4	0	0
2 – 5 years	7	6	1[1])	0
5 –10 years	13	10	2[2])	1[3])
10 –20 years	10	8	2[2])	0
more than 20 years	15	6	9[2])	0

[1]) woman taking oral contraceptive
[2]) receiving no medication
[3]) received antibiotics 5 weeks ago

For the ranges of 120–150, 90–120, 60–90 and 30–60 µg/l the coefficients of variation were 11.6, 7.5, 8.6 and 11.0% respectively.

A random series of urines (with CEA contents between 15 and 100 µg/l) analysed and stored at – 20°C thereafter, were re-run about three months later. Results found in both runs followed the equation Y (first run) = 0.9929x + 4.07; r = 0.9948. A comparable series run about a year later showed Y (first run) = 1.164x – 8.19; r = 0.92721.

Later, the antisera NKI-3 and 6-G-3 became available. The latter was a gift from the *Neville* group (London)

and, like NKI-3, showed a single precipitation line with crude CEA after immunoelectrophoresis.

Seventy-eight urine samples were tested with G-10-2 and 6-G-3. Result: 54 samples had values below 25 µg/l with both antisera. The other samples showed a reasonable correlation (r = 0.8504). Samples re-run with NKI-3 showed a correlation in a range up to about 100 µg/l (figure 11).

At higher values, using NKI-3, dilution of the urine (1:2) in a number of cases showed no parallelism to the standard inhibition curve.

Table 3 compares values obtained in 12 samples with and without preceding perchloric acid treatment for 15 minutes. Perchloric acid treatment was followed by dialysis against twice distilled water and phosphate buffer.

It will be noted that perchloric acid treatment reduced the urinary CEA content in most cases. The presence of tumour or urinary infection seemed to be of no significance.

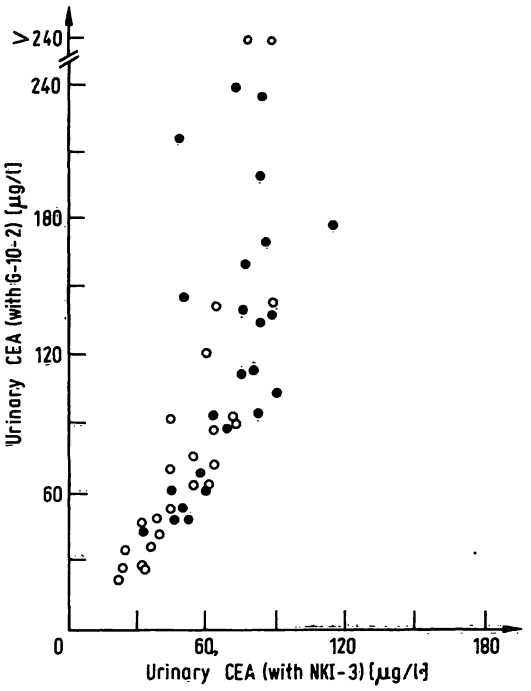

Fig. 11. Comparison of urinary CEA levels measured in undiluted (•) and 1:2 diluted (○) samples with anti-CEA antiserum G-10-2 and with anti-CEA antiserum NKI-3.

Discussion

Choice of assay

A survey of different systems for measuring CEA has been given by *Fleisher* et al. (29). Disadvantages of the zirconyl-gel assay (30), such as large sample volumes, perchloric acid extraction of the samples and subsequent immediate prolonged dialysis which requires large volumes of carefully deionized water, led us to look for less laborious procedures for measuring large series. The direct assay according to a modified *Egan* procedure (15) looked promising as such.

In the indirect assay, the perchloric acid may alter the immunochemical nature of CEA and thus the ability to react with anti-CEA antisera. This is not a hypothetical question in view of the results of our urinary CEA measurements (tab. 3), which indicate that for such measurements perchloric acid treatment should be omitted. The data in figure 9 seem to indicate that perchloric acid treatment of sera does not influence the test result, since the approximately 15% lower values with the zirconyl-gel assay can be explained by endos-

Tab. 3. CEA levels (µg/l) with and without preceding extraction (for 15 minutes) with 1 mol/l perchloric acid (PCA)[1].

	antiserum								tumour present	urinary infection present
	G-10-2				NKI-3					
	undiluted		diluted 1:2		undiluted		diluted 1:2			
Sample	+ PCA	– PCA	+ PCA	– PCA	+ PCA	– PCA	+ PCA	– PCA		
1	–	42	–	–	–	31	–	≤ 30	+	–
2	66	140	–	65	–	75	–	54	+	–
3	160	260	90	141	≥ 100	≥ 100	66	≥ 100	+	–
4	–	235	–	92	–	84	–	70	+	–
5	106	113	–	50	80	63	–	36	+	–
6	66	138	–	76	–	87	–	53	–	+
7	–	88	–	40	68	68	–	39	–	+
8	68	210	–	122	–	83	–	59	+	+
9	130	290	–	145	≥ 100	≥ 100	70	88	+	+
10	–	93	–	48	–	72	–	ND[2])	+	+
11	ND[2])	103	62	52	104	89	–	44	+	+
12	114	135	54	61	66	82	–	59	+	+

[1]) Test results were multiplied by 2, accounting for two-fold increase of volume by adding perchloric acid. Results ≤ 30 were not multiplied and are therefore not inserted in the table.
[2]) ND = not determined.

motic increase of volume during dialysis. No significance should be attached to some discrepancies shown in figure 9 since the large sample volume required for the zirconyl-gel assay (5 ml) made it impossible to confirm these by repeating the assay by either of the two methods. On the other hand, in opting for either the direct or the indirect assay it should be considered that the incidence of false elevated values is not lower with assays based on perchloric acid extraction than with direct assays (31).

Absorption of anti-CEA antisera

Where most investigators used absorbed antisera (3, 7, 21, 32, 33, 34, 35, 36) and judged the specificity of the antiserum by gel diffusion techniques, we raise two critical remarks:

a) The presence or absence of certain precipitation lines after gel diffusion is not a completely valid criterion for mono-specificity in the radioimmuno-assay of the antiserum tested. Conditions such as concentrations prevalent in the radioimmuno-assay are totally different from those in gel diffusion.

Cross-reacting antibodies such as anti-non-specific cross reacting antigen-antibodies may be present and visible in immunodiffusion but diluted out in the radioimmuno-assay system to such a degree that they have no effect on the test results. In gel diffusion the immune complex, if formed, may be too weak to become visible.

b) In view of the abundance and diversity of materials used in absorbing-out (for a review see l.c. (35)), the affinity of anti-CEA antibodies to CEA may very well be decreased by non-specific interactions or by CEA present in the materials.

Indications of such interaction can be found in references 33 and 36 and in this study (fig. 7). We intentionally did not absorb-out NKI-3 since neither anti-non-specific cross reacting antigen antibodies in the antiserum nor blood group substances in 2SDI could be detected under the conditions of the radioimmuno-assay (fig. 6, precipitation experiments with blood group antibodies and results with A-rich sera).

Identification of 2SDI

The amino-acid and carbohydrate analysis data on 2SDI (tab. 1) suggest that we are working with chemically pure material. A more complex question is whether 2SDI is immunologically CEA or related to CEA. Similar behaviour of two different CEA preparations in their binding curves with the same antiserum is not a proof of complete immunological identity but can lead to the conclusion that both preparations have *some* antigenic groupings in common. However, testing several CEA preparations (see fig.'s 2–4) from different laboratories in comparative experiments with 2SDI can contribute to answering the above question. The fact that identical shapes and identical or nearly identical slopes of binding curves were obtained when comparing 2SDI with CEA from different groups, leaves little doubt that 2SDI possesses the same antigenic groups as these four preparations. In addition, the superimposability of the curves (except for fig. 2) implies that this common immunological identity can be related to a weight basis and consequently that we are working with essentially the same antigen. Small antigenic differences may exist but since there are no other criteria for defining CEA, we conclude that 2SDI is real CEA. An indication of the existence of an antigenic difference can be found in figure 4a, showing less complete inhibition of labelled 2SDI by higher concentrations of CEA_{Du}.

Relativity of CEA levels

Several factors which can cause false CEA levels and which in our opinion have not received due attention in the literature, will now be discussed. Purification of CEA by column chromatography involves elution by phosphate buffer followed by dialysis and lyophilisation of the fractions which possess CEA activity.

It is of utmost importance that dialysis is continued till complete removal of phosphate salts.

In the preparation of 2SDI, dialysis was therefore continued until the sodium content of the dialysate was consistently zero. CEA preparations with different salt contents will yield parallel but not superimposable curves in the case of immunological identity. This might explain figure 2. Secondly, let us consider the problem of the selection of normal sera for the preparation of the standard inhibition curve. A small percentage of apparently healthy persons has elevated values, mostly in the range of 3–10 µg/l (16, 32, 37–40). It is therefore of great importance to ensure that a normal serum is applied with the lowest, if possible zero, CEA content to avoid alterations in measured CEA levels due to changes from one batch of normal serum to another. The problem of varying results has been pointed out by *Kupchik* et al. (35). We agree with these authors that the use of a strict cut-off level of 2.5 µg/l between normal and elevated is no longer universally acceptable. We believe we have solved this problem by introducing the "standard pool" as described in "Comments". In this approach the selected normal serum is defined by the standard pool. This provides an accurate means of consistently maintaining a zero base-line, which is a prerequisite for long-term follow-up studies. Distributions of normal sera thus defined, and their incorporations into different assay systems would facilitate comparison of results from different laboratories. We feel that, as a reference material, normal sera checked against a standard pool are needed *as much* as reference CEA preparations. Statements by laboratories that a value of, say, 8 µg/l or higher in patients with colonic carcinoma indicates a poor prognosis (41) have limited value for

other institutes unless a comparative evaluation is made by exchange of normal serum and standard pool. In this connection we might mention another problem. Elevated CEA levels have been found in 14 per cent of 110 chronic cigarette smokers (42) with a direct assay and in 19 per cent of 620 smokers using the zirconyl gel assay (38). The assays take 5 and 2.5 μg/l as the lower limit of positivity, respectively. Smoking habits were not elucidated in these reports. Our results seem to indicate that smoking habits rather than age are related to elevated serum CEA levels. Assessment of limits of CEA levels in healthy smokers again is meaningful to other laboratories only after exchange of normal serum and standard pool. The concentration of the components of the antibody population directed against different antigenic groups may change at increasing dilution of the antiserum to such an extent that some will begin to play a minor role in the radioimmuno-assay. At widely different dilutions, therefore, there is a possibility of obtaining more or less different values for the same samples. To minimize problems in this field we have used, throughout, antisera at dilutions giving about three-quarters of the maximum binding. A complex situation may still arise when two different antisera with comparable percentage of binding differ in their distribution of antibodies. Let us consider the presence of an antigen in the sample possessing a determinant with a low affinity for some groups of the antibody population. High concentrations will not result in completely decreased binding of the labelled antigen as compared with the standard inhibition curve. Consequently the antigen will be quantitated too low. The diluted (e.g. 1:2) sample may give a value higher than calculated and possibly close to the value of the undiluted sample. These influences can manifest themselves in the comparison of two antisera. We believe that the discrepancy in urinary CEA levels measured with NKI-3 and G-10-2 implies that NKI-3 has less antibodies (or antibodies with less affinity) directed against a determinant of urinary CEA present predominantly in urine samples with large amounts of CEA (The 2SDI preparation might bear this determinant as well). The data suggest the possibility that urinary CEA is more heterogeneous than colonic CEA. The sensitivity of CEA in urine to perchloric acid treatment (tab. 3) and work reported by *Neville* et al. (43) support this. The question arises as to which antiserum can be used in follow-up studies of bladder carcinoma patients. The most important point in the management of patients is that the change from normal to moderately increased levels, or vice versa, is identified. Each of the three antisera tested can be effectively used for this purpose. *Neville* et al. (43) have advocated the use of an antiserum raised against purified urinary CEA. As long as such an antiserum is not available we must apply criteria valid for antisera used in the radioimmuno-assay of CEA.

Closing remarks

Problems concerning the antigenic specificity of CEA and cross-reacting relatives remain unsolved. A comparative study as presented in this report is necessary to establish whether a definite CEA preparation or anti-CEA antiserum can be used in clinical studies.

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